CHAPTER V

TISSUE CULTURE AND BIO-PRODUCTION OF BIOCHANIN A

5.1 Introduction

The indiscriminate exploitation of medicinal herbs for bio-active compounds has threatened a number of highly valuable ones. Hence the necessity to develop sustainable bio-production of the active compounds through in vitro plant cell and tissue culture technology has become the need of the hour. This technology will eliminate the risk due to environmental factors (drought, floods, etc.), diseases and chemical fertilizers resulting in uncontrolled variations in the crop yield and secondary metabolites content. Further handling problems and storage loss noticed in conventional agriculture can also be avoided by use of this technology.

Survey of literature about the production of secondary metabolites through tissue culture methods shows that these methods have the partially replaced utilisation of live plants. A few examples are Shikonin from *Lithospermum erythrorhizon* cell cultures\(^{196}\), Berberine from *Coptis japonica* and *Thalictrum minus* cells by Mitsui Petrochemical Industry, Japan; Purpurina from *Rubia akane* and Ginsenoides from *Panax ginseng* by Nitto Denko Corporation, Japan; paclitaxel by Samyang Genex Co., South Korea and Phyton Biotech GmbH, Germany\(^{197,198}\) etc.

Many strategies are being proposed to improve yields of secondary metabolites in plant cell cultures. Screening and selection of high yielding cell lines, optimization of growth and production media, induction of stress, precursor feeding, immobilization of cells and enzymes, bio transformations are important factors studied. Newer approaches like
culturing of differentiated systems like shoots, roots and hairy roots, elicitation and metabolic engineering have been suggested\textsuperscript{199}. It has now been necessitated due to the same reason that the study of phytochemistry needs to be supplemented by research methodologies to produce these phytochemicals through tissue culture studies. These will make the drug discovery from herbs a complete program thereby answering ecological issues too.

5.2 Present study

The present investigation aims at studying the possibility of bio production of Biochanin A, a very potent anti-cancer drug isolated from various parts of \textit{D. sissoides} by tissue culture of its seeds.

The establishment of tissue culture protocol for the production of secondary metabolites requires step wise standardization methods given below.

To initiate and establish a tissue culture system, the first and foremost requirement is deriving a suitable protocol for the achieving clean cultures, without any contamination. The importance of surface sterilization is repeatedly being stressed by many researchers\textsuperscript{200}. However, it is very difficult to obtain sterile plant materials completely free from contamination. Therefore, it is of prime importance to find safe sterilization agent that can remove the fungus and bacteria from the explant tissue.

Suitable tissue culture system is the key for successful \textit{in-vitro} production of secondary metabolites. The reason being many a times secondary metabolite production is differentiation dependent which is controlled by hormones. A few reports which explain the similar concept
are below. Forkolin bio-production in *Plectranthus barbatus*. Culture by Mersinger have reported absence of forskolin and other labdane diterpenoids in suspension cultures cultivated in 2,4-D and KN medium. However a medium containing IBA trigger the biosynthesis. This may be due to the initiation of root primordia on auxin containing medium, developed a high forskolin yielding cell suspension cultures derived from callus and explained the reason for the production of forskolin in tissue cultures. Krombholz established root cultures of *Plectranthus barbatus* biosynthesing forskolin. Sen et al. on the other hand demonstrated the production of forskolin in shoots and caulogenic callus but not or only in traces in rhizogeneic callus and root cultures. The above discussed reports clearly indicate the need for developing protocols for various differentiated and un-differentiated cultures and screen them for secondary metabolite production. Hence, attempts were made to initiate and establish callus cultures, shoot cultures, root cultures.

Nature of explants plays a significant role for successful establishment of tissue cultures. Callogenesis implies an initial stage of growth and differentiation from the parental tissue. To establish callus cultures, the initial tissue used is a fundamental factor in order to achieve the desired response. Explant dependent response has been published in *Erysimum scoparium* by Perez Frances et al.

The present work was carried out with the following objectives,

a) To optimize protocol for initiation and establishment of clean mother cultures

b) To screen different explants for high percent of callogenesis

c) To evaluate different media and plant growth regulators for efficient callogenesis.
d) To pursue phytochemical evaluation and identification of Biochanin A in callus cultures.

e) To Initiate and establish suspension cultures.

f) To conduct phytochemical evaluation and identification of Biochanin A in suspension cultures.

5.3 Materials and Methods

Plant materials: Fresh seeds of D.sissoides collected and were stored in refrigerated condition and used for studies at the lab.

Media preparation: In the present investigation, the nutrient media used were MS medium, B5 medium and LS medium. The composition of the different media used are given in Table 5.1.

Table 5.1 Composition of MS, B5, and LS Media

<table>
<thead>
<tr>
<th>Component</th>
<th>MS (mg/l)</th>
<th>B5 (mg/l)</th>
<th>LS (mg/l)</th>
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<tbody>
<tr>
<td>NH4NO3</td>
<td>1650</td>
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<td>-</td>
</tr>
<tr>
<td>KN03</td>
<td>1900</td>
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<td>FeSO4.7H2O</td>
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Vitamins and Organics

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</tr>
<tr>
<td>Glycine</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

5.4 Stock solution preparation

Concentrated stock solutions of macronutrients, micronutrients and vitamins specific to each medium were prepared and stored in refrigerator. Stocks were used within one month.

**Auxins** - 2,4-D, 2,4-5T, 2,4-5 TP, NAA, IAA, IBA and Picloram were prepared by dissolving the measured quantity in a few drops of 0.1 N NaOH and made up to the known volume with distilled water and stored in refrigerator.

**Cytokinins** - BAP and KN were prepared by dissolving required quantity of compound in few drops of 0.1 N HC1 and then made up to the known volume with distilled water and stored in a refrigerator.

5.5 Carbon source

Analytical grade Sucrose at 3% level was used as carbon source in all the experiments.

5.6 Gelling agent

Agar at 0.8% concentration was used in all the experiments as a gelling agent except for liquid media.

The medium was freshly prepared as and when required by drawing appropriate amounts of stock solutions. After adding 3% sucrose the volume of the media was made up lesser than required volume. The pH of the medium was adjusted in between 5.6 and 5.8 using 0.1 N HC1 or 0.1 N HC2.
NaOH before making up to fixed volume. Agar (0.8 %) was added and boiled before making up 30 ml of molten medium was dispensed into each bottle. These bottles with medium were then autoclaved at 15 lb/in. for 20 minutes. After steam sterilization, the media in the bottles were allowed to gel. In case of liquid media, the pH was adjusted to 5.7 and autoclaved without adding agar.

5.7 Preparation of explants

The nodes, interodes, leaves and pods of *D. sissoïdes*, were separately surface sterilized by thoroughly washing with Tween 20 followed by tap water wash. Explants were treated with 0.1% Bavistin for 45 minutes. Later the surface sterilization procedure was carried under aseptic condition in a laminar air flow chamber.

Various other surface sterilization treatments were tried in order to optimize a suitable protocol for obtaining high percentage of clean and healthy cultures. The treatments are as given under:

| T 1 | 0.1 % HgCl₂ for 5 minutes |
| T 2 | 0.1 % HgCl₂ for 8 minutes |
| T 3 | 0.05 % HgCl₂ for 5 minutes followed by 0.1 % HgCl₂ for 6 minutes |
| T 4 | 0.05 % HgCl₂ for 7 minutes followed by 0.1 % HgCl₂ for 3 minutes |
| T 5 | 0.05 % HgCl₂ for 10 minutes followed by 0.1 % HgCl₂ for 3 minutes |
| T 6 | 10% Sodium hyochlorite for 7 minutes |
| T 7 | 8% Sodium hypochlorite for 7 minutes |

5.8 Inoculation

In advance, the cabinet was cleaned thoroughly with 80 % ethanol and required materials like culture bottles with medium were kept in the
chamber and irradiated with ultraviolet light for 40-60 minutes. Later the UV light was switched on and left for 15 minutes before use. During the course of inoculation and between each inoculation, the surgicals were dipped into 70 % ethyl alcohol, followed by heating in a glass bead sterilizer for 1-2 minutes and cooled before use. After treating with the disinfectants, the explants were thoroughly washed with sterile water for four to five times. The surface sterilized explants were aseptically inoculated on to medium. After the completion of sterile operation, the laminar air flow was cleaned and sprayed with 70 % ethyl alcohol.

5.9 Incubation

The cultures were incubated in growth room at a temperature of 25 ± 2°C, 16 hours light and 8 hours dark cycle were maintained for shoot regeneration and under complete darkness was maintained for callus and root cultures.

5.10 Sub-cultures

Regular transfer of cultures was done at monthly intervals. Experimental protocols, treatments and designs followed in the study are described below

5.11 Experimental protocols

In order to screen and identify a suitable explant with callogenic potential, leaves, stem, node, internode and immature seeds of *D. sissoides* were surfaced sterilized by optimized treatment in the earlier experiment and inoculated on to B, basal media fortified with different growth regulators and at varied concentrations. It was found that the callus cultures initiated from immature seeds showed faster growth and were taken up for further studies. These seed cultures were further inoculated on
to MS, LS and B, medium supplemented with 2 mg/l 2,4-D to select best suitable media for callus induction. Various growth regulators like 2,4-D, 2,4-5T, 2,4-5TP, Picloram, NAA, IAA, IB A, BAP and KN individually and in combination were fortified to selected media and cultures were initiated to study the morphogenic responses. Observations were recorded after 1 month. Morphologically different calli were extracted and analysed for the presence of secondary metabolites. The presence of potent secondary metabolite, Biochanin A, in the callus, shoot cultures and root cultures were analysed using TLC.

Cell suspension cultures were initiated from friable callus regenerated from immature embryos and studied for the production of Biochanin A, Media pH was adjusted to 5.7 before autoclaving. About 75 ml of liquid medium was dispensed into 250 ml Erlenmeyer flasks. Autoclaved medium was used. Friable callus weighing approximately 5 gms was transferred to the flasks under aseptic conditions. Cultures were incubated on a rotary shaker at 120 rpm in darkness and temperature was maintained at 25 ± 2° C. After 15 days of incubation cell suspensions were filtered through appropriate sterile sieves to obtain single cells and few celled aggregates which are used as inoculum for sub-culture. Fine cell suspensions were obtained by repeated sub-culturing of callus and removing small clumps at every stage. The cell suspension cultures were maintained on the same medium by sub-culturing at 2 week interval.

5.1.2 Growth studies of suspension cultures

This is the measurement of volume of cells present at any time during the growth cycle. 20 ml of homogenous liquid cell suspension was transferred into graduated centrifuge tube and centrifuged at 3000 rpm for
5 minutes. The total volume of cell pellet was determined. The packed cell volume was expressed as ml of the total volume in the tube. Fresh and dry weight of the cell biomass was also determined. Endogenous and exogenous production of the compound was determined by the methods given at the end of this chapter.

5.13 Phytochemical screening procedure

Both the solid and liquid culture media were examined for the presence of Biochanin A. The culture media were suspended in chloroform and stirred for 10 minutes for the dissolution of Biochanin A in the organic solvent media. The organic layer was separated in a separating funnel and the water layer discarded. Low polar fractions present were removed by extraction of the concentrated mass with ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulphate and was examined by TLC examination.

TLC analysis was performed over standard Merck G265 pre-coated plates. Chloroform: Methanol (9:1, v/v) was used as the solvent system to elute the plate.

5.14 Results and discussion

The present study is mainly focussed on induction of callus, shoots and suspension cultures and production of Biochanin A in the same. The results of the present study were discussed under.

5.15 Optimization of surface sterilization procedure

Among all the surface sterilization procedures tried mercuric chloride resulted in more number of clean cultures when used sequentially, i.e., treatment with 0.05 % for 7 minutes followed by 0.1 % for 3 minutes (T4 treatment given in materials & methods). Mercuric chloride at higher
concentrations resulted in browning and necrosis of the tissue leading to death. When concentrations and treatment timings further lowered, increase in higher levels of contamination was noticed. Sodium hypochlorite though resulted in lesser contaminations, the explants got bleached yielding unhealthy cultures. Hence to establish clean cultures in *D. sissoides*, mercuric chloride was found to be more suitable than sodium hypochlorite. The toxicity of sodium chlorite is being stressed by Zulfiqar *et al*.

5.16 Screening of various explants for exhibiting morphogenetic response

Leaf, node, inter node and immature seeds were surface sterilized by the successful protocol optimized in the above experiment and inoculated on B5 medium supplemented with 2 mg/l 2,4-D. Of all the explants tried immature seeds exhibited best response with 100% frequency, followed by leaf explants. Callus initiated from cut edges of the leaf explants is slow growing with rapid browning and hence not used in further experimentation. To establish callus cultures the initial tissue used is a fundamental factor in order to achieve the desired response. Hence in the present study the usage of immature seeds to initiating tissue cultures is found to be successful and continued with the same in further experimentation.

5.17 Screening of various media

The nature of response and % of response is dependent on type of media used. Legumes in general and trees in particular are difficult to respond. Among the three media used B5 media found to inducing faster friable callus with maximum percent of response. Hence all other experiments were conducted with B5 media and immature seeds. The
morphogenetic response dependent on nutrients supplied to the tissue cultures, Gibson et al. have reportedly said that nutrients function both as substrates for growth and also act as signal triggering cascade of both primary and secondary metabolite events. Lakshmi sita and Raghava swamy have discussed in detail about the media dependent shoot tip necrosis and leaf fall in Dalbergia latifolia\textsuperscript{a}. By manipulating media these researchers proved to control leaf abscission and shoot tip necrosis in Dalbergia latifolia tissue culture.

5.18 Effect of growth regulators

The type and concentration of growth regulator influenced the morphological nature of callus. In general white friable callus is used to induce suspension cultures. Green organogenetic callus is used to induce shoot buds. Medium fortified with auxin though triggered only callus proliferation, the type of auxing induced morphologically varied calli. Immature seeds cultured on B\textsubscript{5} medium fortified with 2 mg/1 2,4-D resulted in greenish friable and nodular callus Plate VIII (Fig. 5.1). whereas the mixed type of the calli which is brown, green and pale yellow was observed from explants inoculated on B\textsubscript{5} medium fortified with 2 mg/1 2,4-5T Plate VIII (Fig. 5.2). Similarly mixed callus (greenish white) was noticed media supplemented with 2 mg/1 2,4-5-TP Plate VIII (Fig. 5.3). Friable callus is suitable for establishing suspension cultures. On media supplemented with 3 mg/1 NAA friable callus was noticed, however interspurced with hard callus also making it unsuitable for suspension cultures Plate VIII (Fig. 5.4). Interestingly complete friable callus was observed on IAA supplemented media, but turned brown in later stages Plate VIII (Fig. 5.5). IBA supplemented media is no good as this growth regulator induced hard brownish callus Plate VIII (Fig. 5.6).
Greenish friable and nodular callus regenerated on B, fortified with 2.00 mg/1 2,4-D

Mixed type of callus (nodular brown, green and pale yellow) regenerated on Bs medium supplemented with 2.00 mg/1 2,4,5 T

Mixed type of callus nodular, greenish and white regenerated on B, medium fortified with 2.00 mg/1 2,4,5 TP

Friable and hard callus regenerated on Bs medium supplemented with 3.00 mg/1 NAA

Friable brown callus regenerated on B, medium fortified with 3.00 mg/1 IAA

Hard brownish white callus regenerated on B, medium with 3.00 mg/1 IBA
Among all the auxins tried, pieloram at 2 mg/1 level exhibited the best response with maximum friability (Plate IX, Fig. 5.7). The white friable callus proliferated on this medium is using auxins in combination of cytokinins is a common phenomenon by tissue culture research to improve the response patterns used to initiate and establish suspension cultures.

In the present study, the combination of auxin cytokinin and antioxidants were also employed to reduce browning and increase friability. Among various combinations tried, 3 mg/1 NAA and 1 g/1 PVP supplemented media (Plate IX, Fig. 5.8) exhibited relatively better response. Browning of the callus was successfully controlled on these two media. The callus induced on B5 medium supplemented with 2 mg/1 2,4-D and 1 mg/1 BAP regenerated. Friable callus but brown in colour (Plate IX, Fig. 5.10). The presence of BAP along with 2,4-D turned the callus green and nodular indicating organogenesis (Plate IX, Fig. 5.11).

The callus thus developed was transferred to cytokinin supplemented media for inducing organogenesis. The green nodular callus was utilized for induction of shoot buds, whereas the white friable callus was used to induce suspension cultures. Of all the calli, the cultures induced on pieloram containing media induced callus suitable for suspension cultures. The necrosis and browning of the callus was successfully controlled by adding PVP an antioxidant to the media. The friability of the callus was retained by regular subcultures on the same media. Callus cultures induced on all other auxins with an exception of pieloram and NAA either individually or in combination were exhibiting necrosis or browning either initially or at later stages. Hence in conclusion, it could be assumed that pieloram either individually or in combination...
Plate IX

Fig. 5.7  White Friable callus regenerated on medium supplemented with 2.00 mg/l picloram

Fig. 5.8  White Friable callus regenerated on medium supplemented with 3.00 mg/l NAA + 1 g/l

Fig. 5.9  White Friable callus regenerated on B₅ medium fortified with 2.00 mg/l 2,4-D + 1 g/l PVP

Fig. 5.10 Friable brownish callus regenerated on B₅ medium supplemented with 2.00 mg/l 2,4-D + 1 mg/l BAP

Fig. 5.11 Nodular green callus regenerated on 3.00 mg/l BAP + 1 mg/l 2,4-D
was suitable to induce friable callus cultures, where as NAA and BAP could be used to induce organogenesis.

The type and nature of callus depends mainly on type and concentration of growth regulator used. Development of brown callus has been reported by Reddy et al. in *Coleus forskolii*. There are many reports of successful usage of Pieloram as an auxin like agent along with BAP or KN for effective induction of regenerative calli in a number of plant species\textsuperscript{216}. This is in agreement with the present study, where pieloram is found to be more effective to induce friable callus in *D. sissoides*. One of the major problems during tissue culture of tree species is exudation of phenolics from the tissues which either result in browning or necrosis\textsuperscript{217}.

The browning can be controlled to some extent by using antioxidants like PVP and activated charcoal in media. In the present investigation PVP supplemented media successfully induced friable callus without any browning. Usage of PVP to avoid browning has been studied by Abdelwahd\textsuperscript{218}.

Induction shoots from callus on cytokinin supplemented media. The concentration of harmones is given in Plate X & Plate XI (Fig. 5.12, 5.13, 5.14, 5.15, 5.16 & 5.17) and (Fig. 5.18, 5.19, 5.20, 5.21, 5.22, 5.23). Induction shoots from callus on cytokinin supplemented media (0.1 mg/l KN). The cytokinin dependent shoot regeneration in legumes is reported by Buising et al\textsuperscript{m}.

**5.19 Analysis of different calli for the presence of Biochanin A**

White friable calli, green nodular calli, organogentic calli and calli grown on different plant growth regulators the biosynthetic potentiality and positive for the Biochanin A whereas brown necrotic calli was found
Fig. 5.12 *Induction shoots from callus on cytokinin supplemented media (ISCCSM) 0.10 mg/1 KN*

Fig. 5.13 (ISCCSM) 0.25 mg/1 KN

Fig. 5.14 (ISCCSM) 0.50 mg/1 KN

Fig. 5.15 (ISCCSM) 1.00 mg/1 KN

Fig. 5.16 (ISCCSM) 0.10 mg/1 BAP

Fig. 5.11 (ISCCSM) 0.25 mg/1 BAP
Plate XI

Fig. 5.18 Induction shoots from callus on cytokinin supplemented media (ISCCSM) 0.50 mg/1 BAP

Fig. 5.19 (ISCCSM) 1.00 mg/1 BAP

Fig. 5.20 (ISCCSM) 0.50 mg/1 KN + 0.10 mg/1 BAP

Fig. 5.21 (ISCCSM) 0.50 mg/1 BAP + 0.10 mg/1 KN

Fig. 5.22 (ISCCSM) 1.00 mg/1 BAP + 0.50 mg/1 KN

Fig. 5.23 (ISCCSM) 1.50 mg/1 BAP + 0.50 mg/1 KN
to be negative to Biochanin A. The photographs of TLC plates and description are given in Plates XII & Plate XIII.

5.13 Initiation and establishment of suspension cultures

Friable callus was transferred to B, liquid medium supplemented with 2 mg/1 Picloram and incubated on a shaker. Cell suspensions were grown for 15 days. The cells and suspensions were subjected to growth studies. The cell suspension was subjected to packed cell volume identification by centrifuging the suspension. The pellet and spent media were subjected to extraction and analysis. Both exogenous and endogenous production of Biochanin A were noticed.

From the above experiments it has been clearly proved that the same protocol can be improvised to bio-synthesise Biochanin A, a very potent anti-cancer drug which is otherwise extractable only by destruction of tonnes of biomass which can lead to even extinction of this species.
PLATE XII

S1 - Organogenetic callus, grown on BAP
S2 - Standard of Biocharin - A
S3 - Organogenetic callus grown on KN

S1 - Friable callus (white)
S2 - Brown callus
S3 - Standard
S4 - Green callus
PLATE XIII

- S1: Callus grown in media containing 2,4-D
- S2: Spot is callus grown in media containing 2,4-D
- S3: Spot is callus grown in media containing 2,4-D TP
- S4: Standard
- S5: Spot is callus grown in media containing pindoram
- S6: Callus grown in media containing NAA
- S7: Callus grown in media containing IAA
- S8: Spot is callus grown in media containing IBA

- S1: Standard Biochanin A
- S2: Callus cultures
- S3: Spent medium of cell cultures
- S4: Cell biomass of suspension cultures